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In breast cancers with *erb*B-2 overexpression abnormal cell proliferation is caused by the extremely active tyrosine kinase activity and resulting high level of signal transduction. An early and important intermediate in this signalling is the adaptor protein Grb2. This application proposed to design and test novel Grb2 inhibitors. This analysis will have important implications for translational cancer research by testing Grb2 inhibition as a target for therapy using compounds of defined biochemical activity. The support from USAMRMC for the peptide based studies has led to development of modified peptidomimetics and small molecule inhibitors and successful funding from the Komen Foundation for Breast Cancer Research. The results from the peptide Grb-2 inhibitors study provided an important proof-of-concept for an approach that may generate specific, potent SH2 antagonists as clinical candidates in the near future. This type of SH2 antagonists might have a better therapeutic potential to be used either by themselves or in combination with other conventional chemotherapeutics in the treatment of breast cancer.

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Introduction

In breast cancers with *erb*B-2 overexpression abnormal cell proliferation is caused by the extremely active tyrosine kinase activity and resulting high level of signal transduction. An early and important intermediate in this signalling is the adaptor protein Grb2. This application proposed to design and test novel Grb2 inhibitors. This analysis will have important implications for translational cancer research by testing Grb2 inhibition as a target for therapy using compounds of defined biochemical activity.

Our approach was based on two discoveries. First, Dr. King and his associates have identified two peptides that are capable of binding to Grb2 and blocking its function. These peptides act to block the SH2 and N-terminal SH3 domain of Grb2. Second, publications report methods that facilitate introduction of peptides into cells. These studies show that membrane transit can be effected by fusion with a peptide sequence derived from either the signal sequence of protein that naturally crosses cell membranes or a sequence from the antennaepedia protein. Dr. King in his original application proposed to unite these approaches for the generation and testing of peptides capable of blocking growth factor signal transduction in human cancer cells.

The overall objective was to evaluate the phenotypic consequences of blocking Grb2 mediated signal transduction on the malignant phenotype of cancer cells that overexpress growth factor receptors such as p185*erb*B-2. We propose to measure the effectiveness of peptides in interrupting the targeted biochemical interaction in cell lysates and in whole cells and to correlate this intervention with changes in cell growth rate, cell migration, and anchorage independent growth. Since Grb2 signalling is part of diverse signal transduction pathways, we will also investigate whether Grb2 inhibitors effect cells that have no evidence of *erb*B-2 or other growth factor overexpression. In this way we test the hypothesis that Grb2 inhibitors have preferential activity for cancer cells with overexpression or activation of growth factor receptor tyrosine kinase activity. The validation of this hypothesis enhance the rationale for discovery, design, and pre-clinical testing of Grb2 inhibitors as cancer therapies. The support from USAMRMC for the peptide based studies has led to development of modified peptidomimetics and small molecule inhibitors and successful funding from the Komen Foundation for Breast Cancer Research.

The results from the peptide Grb-2 inhibitors study provided an important proof-of-concept for an approach that may generate specific, potent SH2 antagonists as clinical candidates in the near future. This type of SH2 antagonists might have a better therapeutic potential to be used either by themselves or in combination with other conventional chemotherapeutics in the treatment of breast cancer.

Specific Introduction for This Report.

Protein-protein interactions are involved in the regulation of virtually all aspects of physiological processes, for example, cellular signaling, and metabolic and transcriptional activation in cells (1, 2). Grb2 is an intracellular adaptor protein that consists of one SH2 domain and two SH3 domains, and mediates cellular signaling on growth factor (GF) receptor activation (3). Its Src homology 2 (SH2) domain binds to specific tyrosine phosphorylated motifs on activated GF receptors such as

EGFR and members of *erb*B family, and this leads to downstream activation of the Ras signal pathway which is highly relevant to a number of diseases including breast cancer (4, 5). Therefore, blocking the interaction between the phosphotyrosine-containing activated GF receptor and Grb2-SH2 domain provides a promising therapeutic target for development of new antitumor agents (6, 7). However, phosphotyrosine residue still serves as a critical recognition determinant for effective inhibitors binding to Grb2-SH2 (8-11). In the context of affording more bioavailability and specificity to binding (11-13), we developed a novel non-phosphorylated thioether cyclic peptide ligand (termed as G1TE, **1**) based on the original 1997 discovery of a phage library derived lead peptide (designated as G1) which bound to the Grb2-SH2 protein with 10-25 μM affinity, and was comprised of a 9 a.a. long sequence motif, $\mathbf{E^1\text{-}L\text{-}Y^3\text{-}E\text{-}N^5\text{-}V\text{-}G\text{-}M\text{-}Y^9}$, flanked by 2 terminal disulfide linked cysteines (14, 15). G1 was shown to lack binding propensity in the disulfide reduced open chain form, and it was found to be inactive under physiological conditions in inhibiting Grb2 / p185$^{erb\text{B-}2}$ association. On the other hand G1TE is redox stable, it exhibited equipotent binding affinity to G1, and was demonstrated to inhibit the association of the Grb2 protein with the growth factor receptor p185$^{erb\text{B-}2}$ in cell lysates derived from the breast cancer cell line MDA-MB-453 (14). Ala mutation studies on G1 indicated that essentially all amino acids, except Gly^7 in the peptide were necessary for retention of binding affinity (14), and Asn^5 and the unphosphorylated Tyr^3 were particularly important. G1 and G1TE define a new type of SH2 domain binding motif in a pTyr independent manner, but, at the same time our initial findings demonstrated that G1 requires a **YXN** sequence similar to that found in natural pTyr-containing ligands. We were intrigued to examine the functional importance and structural requirement of Tyr in particular in the consensus sequence of G1TE. We describe here a thorough study of the effect of incorporating various Tyr homologs and analogs, including tyrosine phosphate mimics on the binding affinity of the prototype peptide (Fig. 1). These studies provide an improved understanding of the molecular binding mechanism of this novel agent and suggest new strategies for designing potent non-phosphorylated inhibitors of Grb2-SH2 domain. We have submitted the annual report in August 2000, and received the acceptance from the UDMRMC Research Data Management on January 4 2002. Since the funding ended in July 31, 2001, this report will serve the final report for the entire funding period. However, we are continuing collaboration with Dr. Peter Roller, Laboratory of Medicinal Chemistry, National Cancer Institute, National Institutes of Health, on the modified peptides. The work on the small molecule inhibitors for the same target, Grb-2, is being pursued with Dr. Terry Burke, at the same institute with the funding from the Susan G. Komen Breast Cancer Foundation.

Body of Report

There were three technical objectives in the original proposal. The technical objective 1 was to generate cell permeable peptide inhibitors of Grb2. This has been done in collaboration with Dr. Peter Roller at Laboratory of Medicinal Chemistry of NCI. However, the initial fusion peptides made from the G1TE with a sequence from the antennaepedia protein failed due to the solubility and cell penetration problem. This may be peptide sequence specific problem since the other fusion peptides with the antennaepedia peptide worked well in other systems. We therefore made alternative efforts through a thorough study of the effect of incorporating various Tyr homologs and analogs, including tyrosine phosphate mimics on the binding affinity of the prototype peptide

G1TE (Fig. 1). We have made twice more than the originally proposed peptide inhibitors. As shown in Tables 1 and 2, 14 peptides have been synthesized and their sequences were characterized by mass spectrometry.

The technical objective 2 was to determine the biochemical activity of cell permeable peptides. These include testing co-immunopreciptation inhibition method, testing effects of cell permeable peptides on co-immunopreciptiation of SOS1 and p185*erb*B-2 when peptides are added to cell lysates, determining IC_{50} of inhibition in cell lysates, testing effects of cell permeable peptides on co-immunopreciptiation of SOS1 and p185*erb*B-2 when peptides are applied to intact cells and testing time course of activity of cell permeable peptide activity for Grb2 inhibition.. All of these objectives have been accomplished and are described in the following body of report.

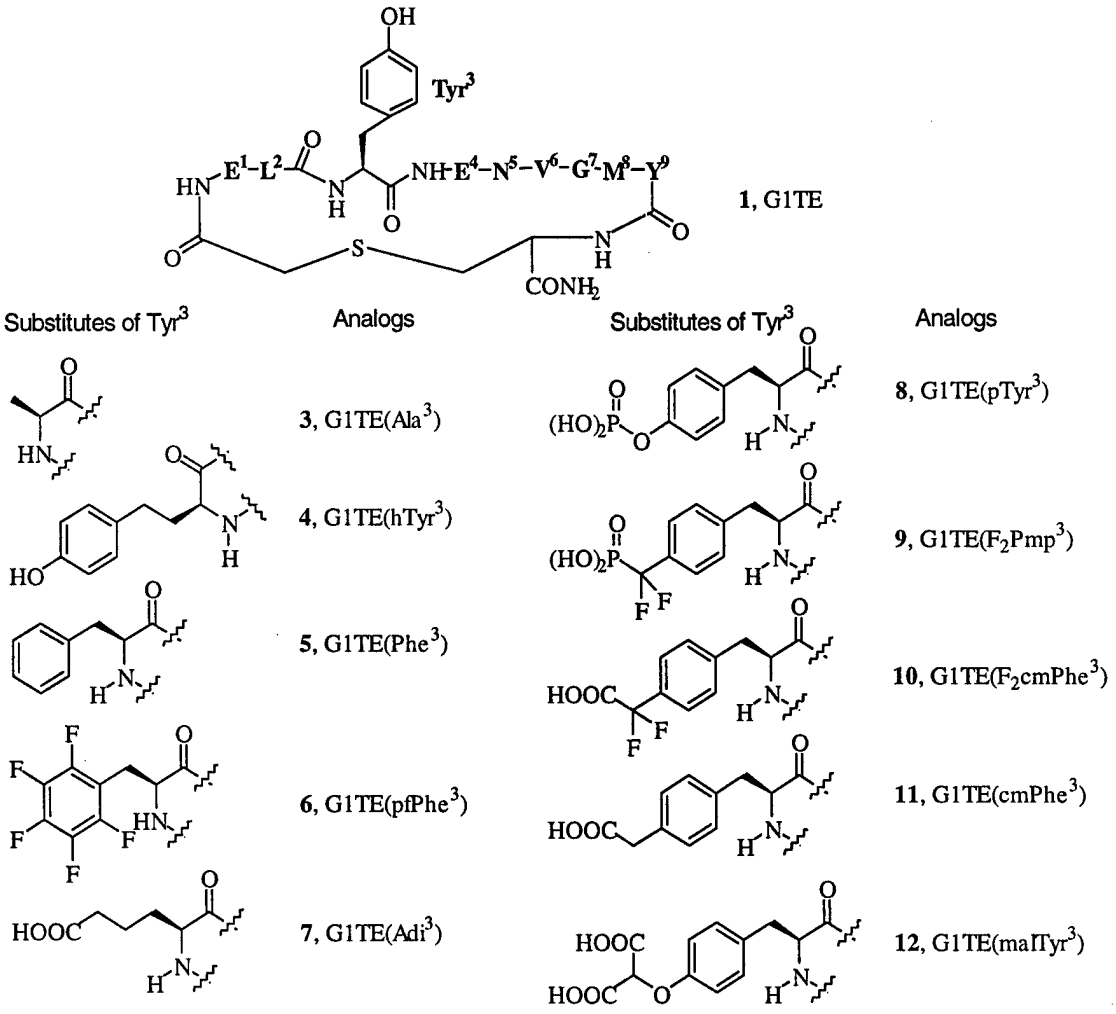


Figure 1. The structures of Tyr analogs and variants substituted at position 3 in G1TE.

The technical objective 3 was to determine effects of cell permeable peptides on cancer phenotype. This objective has been accomplished and is also described in the body of this report.

Design and Synthesis of Modified Grb2 Inhibitors. The synthesis of the cyclic thioether peptides **1-14** was carried out in a convenient manner, similar to that previously reported (16, 17). Briefly, the linear peptide was synthesized on solid phase on a PAL amide resin with an ABI 433A peptide synthesizer utilizing FastMoc chemistry. After removing the N^{α}-Fmoc group with 20% piperidine/DMF, the resin-bound protected peptide was N-terminally chloroacetylated by $(ClCH_2CO)_2O$, which was prepared by mixing 0.5 M $ClCH_2COOH$/DCM and 0.5 M DCC/DCM for 1 h at RT, and filtering off the precipitated DCU. Chloroacetylation was carried out for a duration of 6 hrs at RT, or until the Ninhydrin Test proved to be negative. The open chain peptide was cleaved from the resin by using TFA containing 2.5% each (v/v) of triethylsilane and deionized water (2 h). For isolation of the product, two-thirds of the cleavage reagent mixture was evaporated under N_2 and the mixture triturated in ice-cold ether. For cyclization the precipitated crude peptide was dissolved in 50 mL of water and added dropwise into 100 mL of aqueous solution, which was adjusted to pH 8~9 with triethylamine, repeatedly. Under the basic conditions the N-chloroacetylated linear peptide cyclized spontaneously by intramolecular nucleophilic displacement of the chloro group by cysteine thiol. The final product was purified by RP-HPLC, and the identity was assessed by amino acid and mass spectral analyses (see Table 1).

Table 1. The physicochemical data of peptides **1-14**

Peptide	RP-HPLC[a]	FAB-MS[b]	Amino Acid Analysis
1	R_t = 12.8 min (gradient 20-80% B over 30 min, I)	$(M+H)^+$ 1258.7 (calc. 1259.5)	Asp 1.418(1), Val 1.348(1), Leu 1.345(1), Glu 2.768(2), Gly 1.384(1), Tyr 2.508(2), Met 0.997(1)
2	R_t = 13.9 min (gradient 20-60% B over 30 min, I)	$(M+H)^+$ 1096.8 (calc. 1097.3)	Asp 1.13(1), Val 0.98(1), Leu 1.17(1), Glu 1.78(2), Gly 1.01(1), Tyr 0.65(1), Met 1.01(1)
3	Rt = 12.7 min (gradient 20 ~ 80% B over 30 min, I)	$(M+H)^+$ 1166.0 (calc. 1167.3)	Asp 0.61(1), Val 1.00(1), Leu 1.23(1), Glu 2.05(2), Gly 1.20(1), Ala 1.07(1), Tyr 0.63(1), Met 0.88(1)
4	R_t = 14.1 min (gradient 20-70% B over 27 min, I)	$(M+H)^+$ 1273.8 (calc. 1273.5)	Asp 0.55(1), Val 1.05(1), Leu 1.21(1), Glu 1.93(2), Gly 1.34(1), Tyr 0.73(1), Met 0.85(1)
5	R_t = 15.9 min (gradient 20-80% B over 30 min, I)	$(M+H)^+$ 1244.2 (calc. 1244.5)	Asp + S-CM-Cys 1.14(1 each), Val 1.22(1), Leu 1.34(1), Glu 1.94(2), Gly 1.25(1), Tyr 0.75(1), Met 0.72(1), Phe 1.19(1)
6	R_t = 16.3 min (gradient 20-70% B over 27 min, I)	$(M+H)^+$ 1334.6 (calc. 1334.4).	Asp 1.10(1), Val 0.97(1), Leu 1.15(1), Glu 1.80(2), Gly 1.05(1), Tyr 0.66(1), Met 0.97(1)
7	R_t = 12.8 min (gradient 20-70% B over 27 min, I)	$(M+H)^+$ 1239.6 (calc. 1239.5).	Asp + S-CM-Cys 1.81(1 each), Val 1.09(1), Leu 1.19(1), Glu 1.93(2), Gly 1.09(1), Adi 0.88(1), Tyr 0.84(1), Met 1.01(1)
8	R_t = 13.3 min (gradient 10-70% B over 25 min, I)	$(M+H)^+$ 1339.8 ($calc._{ave}$ 1338.5)	Asp + S-CM-Cys 1.91(1 each), Val 1.13(1), Leu 1.17(1), Glu 1.65(2), Gly 1.20(1), Tyr 1.65(2)*, Met 0.96(1)

9	R_t = 11.0 min (gradient 20-60% B over 30 min, I)	$(M+H)^+$ 1373.6 (calc. 1373.5)	Asp + S-CM-Cys 1.31(1 each), Pro 1.42(1)*, Val 1.21(1), Leu 1.15(1), Glu 1.93(2), Gly 1.19(1), Tyr 0.83(1), Met 0.63(1)
10	R_t = 13.9 min (gradient 20-70% B over 27 min, I)	$(M+H)^+$ 1337.3 (calc. 1337.5)	Asp + S-CM-Cys 1.68(1 each), His 1.77(2)*, Val 1.20(1), Leu 1.32(1), Glu 2.08(2), Gly 1.21(1), Tyr 0.73(1), Met 0.77(1)
11	R_t = 16.3 min (gradient 10-70% B over 27 min, II)	$(M+H)^+$ 1361.8 ($calc._{ave}$ 1362.5)	Asp + S-CM-Cys 1.48(1 each), Val 1.16(1), Leu 1.27(1), Glu 1.97(2), Gly 1.14(1), Arg 1.38(1)*, Tyr 0.69(1), Met 0.77(1)
12	R_t = 13.7 min (gradient 20-70% B over 27 min, I)	$(M+H)^+$ 1302.2 ($calc._{ave}$ 1302.4)	Asp + S-CM-Cys 1.58(1 each), Val 1.13(1), Leu 1.21(1), Glu 1.86(2), Gly 1.17(1), Tyr 0.89(1), Met 0.97(1)
13	R_t = 18.7 min (gradient 10-70% B over 27 min, II)	$(M+H)^+$ 1243.1 (calc. 1243.5)	Asp + S-CM-Cys 1.17(1 each), Val 0.95(1), Leu 1.09(1), Glu 0.94(1), Gly 1.12(1), Ala 0.96(1), Tyr 0.70(1), Met 0.68(1)
14	R_t = 18.5 min (gradient 10-70% B over 27 min, II)	$(M+H)^+$ 1315.6 ($calc._{ave}$ 1315.5)	Asp + S-CM-Cys 1.24(1 each), Adi 0.91(1), Val 1.06(1), Leu 1.13(1), Glu 0.96(1), Gly 1.05(1), Tyr 0.75(1), Met 0.65(1)

[a] HPLC conditions I: Vydac C18 column (10x250 mm); solvent gradient, A, 0.05% TFA in water; B, 0.05% TFA in 90% acetonitrile in water with gradient indicated; flow rate, 2.5 mL/min; UV detector, 225 nm. HPLC conditions II: Vydac C4 column (20x250 mm); solvent gradient, A, 0.05% TFA in water; B, 0.05% TFA in 90% acetonitrile in water with gradient indicated; flow rate, 10.0 mL/min; UV detector, 225 nm.

[b] FAB-MS (unit resolution, glycerol matrix) was performed on a VG Analytical 7070E-HF mass spectrometer.

[c] Amino acid analysis (6N HCl, 100 °C, 24 h) was carried out at the Protein and Carbohydrate Structure Facility (University of Michigan, Ann Arbor, Michigan, USA).

* For peptides containing nonstandard amino acids, additional unassigned HPLC peak was observed.

The PAL amide resin and Fmoc derivatives of standard amino acids were obtained from Perkin-Elmer/Applied Biosystems Division (Foster City, CA, USA). L-homoTyr (hTyr), L-α-aminoadipic acid (Adi) and O-malonyl-L-Tyr (malTyr) were purchased from BACHEM (Torrance, CA, USA) in Fmoc protected form. N^{α}-Fmoc-L-Tyr(PO(OH,Obzl))-OH from NOVAbiochem (La Jolla, CA, USA) was used for the synthesis of phosphotyrosine-containing analogs. Fmoc-L-2,3,4,5,6-Pentafluoro-Phe-OH (pfPhe) was purchased from Synthetech Inc. (Albany, OR, USA). Phosphotyrosyl mimetics 4-carboxymethyl-L-phenylalanine (cmPhe) and 4-carboxydifluoromethyl-L-phenylalanine (F_2cmPhe) were prepared with O-tBu sidechain protection and N-terminally Fmoc protected (18). N^{α}-Fmoc-4-phosphonodifluoromethyl-L-phenylalanine (F_2Pmp) was synthesized with sidechains unprotected (19).

Binding affinity measurement of peptides to Grb2-SH2 domain using Surface Plasmon Resonance (SPR). The competitive binding affinity of ligands for the Grb2-SH2 protein was assessed by using Biacore SPR methods. On a BIAcore 2000 instrument (Pharmacia Biosensor, Uppsala, Sweden). IC_{50} values were determined by mixing various concentrations of inhibitors with the recombinant GST-Grb2-SH2 domain protein and measuring the amount of binding at equilibrium to an immobilized SHC phosphopeptide($pTyr^{317}$), i.e. biotinyl-DDPS-pY-VNVQ, in a manner described previously (14). The biotinylated phosphopeptide was attached to a

streptavidin coated SA5 Biosensor chip, and the binding assays were conducted in pH 7.4 PBS buffer containing 0.01% P-20 surfactant (Pharmacia Biosensor).

Molecular Modeling. The Insight II 97/Discover 3.0 modeling package from Molecular Simulations Inc., San Diego, CA with the cff91-force field was used. The X-ray structure of the KPFpYVNV peptide ligand bound to the Grb2-SH2 was used as a starting geometry (20). The positions of the backbone atoms, and of the sidechain atoms of those residues in the turn region of the reference peptide sequence, -F-pY-V-N-V-, that are identical in G1TE(Adi^1, cmPhe^3) were used as the initial atom positions of the sub-sequence, -L-cmPhe-E-N-V- in G1TE(Adi^1, cmPhe^3), since a turn has been predicted for the related subsequence in G1TE also (21). Then, the remaining residues of G1TE(Adi^1, cmPhe^3) were added to this model. The turn comprising atoms, -CO(cmPhe), Glu, Asn, NH(Val)-, and the protein atoms were kept fixed during minimization and simulated annealing (SA). 75 SA cycles were run with a different random seed for each cycle. In each SA cycle the same minimized starting geometry was subjected to an MD simulation at 2000K for 10 ps and then cooled in 5K decrements to 5K during 195 ps. The final structure was minimized and stored.

The structure with the lowest energy out of the 75 obtained structures served as the starting geometry for three 100 ps MD (NVT-ensemble) simulations at 298 K. Three different random seed numbers were used, and the complex was solvated by a sphere of water molecules with the radius of 22 Å, centered around the Cα of Glu^4 of G1TE(Adi^1, cmPhe^3). During minimizations and simulations, all atoms were held fixed except the ligand, the water molecules within a radius of 18 Å around the Cα of Glu^4 of G1TE(Adi^1, cmPhe^3), and the sidechains of Grb2-SH2 within 6 Å around G1TE(Adi^1, cmPhe^3). The coordinates were saved every 1 ps and minimized by 300 steps of CG-PR. The frame with the lowest energy of the 300 obtained solvated structures was identified and is depicted in Fig. 2.

Inhibition of Grb2 interaction with Tyr-phosphorylated p185(erbB-2). Cell lysates were prepared from serum-treated erbB2 overexpressing breast cancer cells (MDA-MB-453), as described previously (14). Cell lysates were treated with G1TE(Adi^1, cmPhe^3) or control peptide G1TE(Ala^3) at various concentrations for 30 min, and then 500 μg of protein was immunoprecipitated with anti-Grb2 antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) and collected with protein A Sepharose. Immunoprecipitated proteins were separated by SDS-PAGE on 8-16% gradient gels (Novex, San Diego, CA, USA). pTyr-containing proteins were detected by Western blotting using anti-phosphotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY, USA) and visualized with ECL (Amersham, Arlington Heights, IL). Previous experiments have shown that a major tyrosine phosphorylated protein in these cells is the p185$^{erbB\text{-}2}$, which is overexpressed as a consequence of gene amplification (22). To evaluate the equal loading of the proteins, blots were subsequently re-probed with a monoclonal antibody recognizing the total Grb2 protein. A semi-quantitation of the Grb2 associated p185 $^{erbB\text{-}2}$ band was done with densitometer and expressed as percent of control in the untreated cells.

Table 2. Grb2-SH2 Domain Inhibitory Activity of the Peptides **1-14**[a]. Variation in X^3 and X^1.

Compound	Substitute of Tyr^3	Analog term	IC_{50} (μM)
1	Tyr	G1TE	20 ± 5
2	Deleted	G1TE (no Tyr^3)	> 1000
3	Ala	G1TE (Ala^3)	> 1000
4	Htyr	G1TE ($hTyr^3$)	At 1000 μM, 40% inhibition
5	Phe	G1TE (Phe^3)	94.5 ± 10.5
6	PfPhe	G1TE ($pfPhe^3$)	> 1000
7	Adi	G1TE (Adi^3)	600
8	pTyr	G1TE ($pTyr^3$)	0.13 ± 0.01[b]
9	F_2Pmp	G1TE (F_2Pmp^3)	0.88 ± 0.09
10	F_2cmPhe	G1TE (F_2cmPhe^3)	5.7 ± 0.5
11	malTyr	G1TE ($malTyr^3$)	6.0 ± 0.4
12	cmPhe	G1TE ($cmPhe^3$)	1.15 ± 0.05
13	CmPhe (Ala^1 mutant)	G1TE (Ala^1, $cmPhe^3$)	1.65 ± 0.15
14	CmPhe (Adi^1 mutant)	G1TE (Adi^1, $cmPhe^3$)	0.70 ± 0.12

[a]The experiments were performed on a BIAcore 2000 instrument by the method described previously (14). The results represent mean value of at least two independent experiments and are expressed as the concentration at which half-maximal inhibition (IC_{50}) of binding of Grb2-SH2 to biotinylated DDPSpYVNVQ was observed. IC_{50} of reference SHC(pY317) peptide: 1.0±0.2 μM.
[b]This value has previously been reported (23), and is used here as a reference.

RESULTS AND DISCUSSION:

Tyrosine 3 in G1TE is required for Grb2-SH2 binding. - The phage library derived nonphosphorylated cyclic peptides, typified by G1TE, **1**, comprise a unique family of agents binding to the SH2 domain of the intracellular adapter protein Grb2 (14,16). Earlier Ala mutation studies demonstrated that essentially all amino acids are required for good binding affinity, especially Tyr and Asn in the consensus sequence Y^3-X-N^5 in G1TE. Our recent structure / activity studies amply demonstrated that the carboxyl sidechain of Glu in position 1 of G1TE partially compensates for the absence of the phosphate group on Tyr^3 (23). In order to assess the importance of the consensus tyrosine in these peptides and then help design potent non-phosphorylated inhibitors, we synthesized a series of G1TE analogs substituted at the Tyr^3 position and evaluated their binding affinity to the Grb2-SH2 domain with the Biacore binding assays (Table 2). As shown in the table, deletion (**2**) or Ala substitution (**3**) of Tyr^3 eliminates the inhibitory activity, which strongly suggests that Tyr^3 is a very important determinant for high affinity binding of the non-phosphorylated peptide for the Grb2 SH2 domain. But just extending the side-chain of Tyr with one CH_2 moiety, i.e. substitution of Tyr with homotyrosine (hTyr) substantially diminishes the binding (**4**), which indicates the binding affinity is very sensitive to the positioning of phenyl ring or the phenolic hydroxyl group of the sidechain. Comparison of the potency loss on Phe (**5**, IC_{50} = 94.5±10.5 μM) and the non-aromatic α-amino-adipate (Adi)

replacements (**7**, IC_{50} = 600 μM) for Tyr^3 further confirms that important sidechain interactions can be achieved only when the functional sidechain is positioned precisely to fit the binding pocket of the Grb2-SH2 domain. The Phe^3 mutant still sustains moderate binding to SH2, and this points out the functional importance of the aromatic sidechain itself at position 3, regardless of the loss of polar substituents. In comparison, the pentafluoro-Phe replacement (**6**) abolishes the binding. Both the increase in hydrophobicity and the reduced electron density of the phenyl ring as a result of the strong electron-withdrawing fluorine substituents appear to disfavor association within the protein binding pocket (24). Remarkably, our molecular modeling studies of nonfluorinated G1TE peptide / Grb2-SH2 complex, as well as the X-ray structure of the phosphopeptide / Grb2-SH2 complex (20) indicate that the polar sidechains of Arg67 and Lys109 of the protein are in a position to form aromatic-π / cation interactions with pTyr or Tyr of the peptides. It is known that fluorination of the aromatic ring disrupts such interactions (24). These results farther corroborate the importance of cation / π interactions in the Tyr or pTyr binding pocket of Grb2-SH2 domain and presumably in the related Src-SH2 protein also (25). We have carried out *ab initio* calculations (Gaussian 94, HF/6-31G**, in vacuum) to estimate the difference in interaction energy in a simple model system. For benzene/NH_4^+ a favorable geometry was found with the ammonium ion located 3 Å above the center of the plane of benzene ring, with a favorable interaction energy of –15 kcal. In contrast, the analogous single point energy calculation of a complex of geometry-optimized pentafluorobenzene and NH_4^+ showed that this complex was disfavored by 4 kcal. From the results above, we can conclude that optimally positioned phenyl moiety and to some extent the polar phenolic 4-hydroxyl functionality are required for effective binding of this non-phosporylated inhibitor.

[**Figure 2**] **Figure 2. A.** Complex of the cyclic thioether peptide G1TE(Adi^1, $cmPhe^3$), **14**, with the Grb2-SH2 domain protein, based on molecular modeling. *Note*: the solid green stick structure corresponds to the peptide ligand. **B.** Schematic representation of the ionic and/or polar interactions between the –Glu^1-Leu-$cmPhe^3$-Glu-Asn^5- segment of the peptide ligand **14,** based on molecular modeling. **C.** Schematic representation of ionic and/or polar interactions in a complex of the Bcr-Abl phosphopeptide and the Grb2-SH2 domain, based on X-ray sructural information (20). *Note:* not all interactions are indicated.

Tyrosine phosphate mimics in G1TE provide efficient inhibitors of Grb2-SH2 domain interactions. - Predictably, phosphorylation of Tyr^3 in the cyclic peptide, **1,** greatly improved the Grb2-SH2 binding affinity, by a factor of 150 fold (**8**, IC_{50} = 0.13±0.01 μM). However, phosphotyrosine itself is not a desirable building block for *in vivo* active inhibitor design, due to the hydrolytic lability of the phosphate ester toward phosphatases and the poor membrane penetration of the doubly ionized phosphate group.

Our subsequent efforts focus on examining the effect and potential use of phosphotyrosyl mimetics as replacements of Y^3 on the binding efficacy to Grb2-SH2 domain in the context of this novel non-phopshorylated peptide ligand.

F_2Pmp was reported earlier to be a good phosphatase-resistant surrogate for the pTyr residue (26). Incorporation of F_2Pmp into position 3 of **1** results in submicromolar affinity inhibitor (**9**,

IC_{50} = 0.88±0.09 μM), but reduces the potency by 6-fold relative to G1TE(pTyr^3), which was contrary to the finding of F_2Pmp retaining as good binding affinity as pTyr in pTyr-containing short peptide inhibitors of various SH2 domains (26). The reduction in potency of F_2Pmp might be attributed to the loss of interactions with the pTyr ester oxygen which was replaced by a difluoromethylene unit in F_2Pmp, and in our case, the fluorines added in the bridging methylene can not restore the lost interactions, as was previously observed in phosphopeptides (26). Even with the retention of the bridging oxygen, O-malonyl-L-tyrosine replacement for Tyr^3 in G1TE does not remarkably increase the inhibitory activity (**12**, IC_{50} = 6.0±0.4 μM). This might result from non-optimal positioning of the carboxyl groups because of a too long linking side-chain. These results further confirm the notion that the nonphosphorylated cyclic peptide ligand **1** requires more specific conformation and interactions with the binding pocket of the protein, most likely involving the side chains of nearby residues e.g. E^1, Y^3, E^4, N^5 and M^8.

4-Carboxymethyl-L-phenylalanine (cmPhe) and 4-carboxydifluoromethyl-L-phenylalanine (F_2cmPhe) were successfully utilized previously as non phosphorus-containing pTyr mimetics in a high affinity β-bend mimicking platform (27, 28), and in this case, substitutions of cmPhe and F_2cmPhe for Tyr^3 in **1** resulted in the binding potency enhancement by 17-fold (**12**, IC_{50} = 1.15±0.05 μM) and 4-fold (**10**, IC_{50} b= 5.7±0.5 μM), respectively, relative to the parent peptide. The introduction of fluorine into the cmPhe residue reduced the SH2 domain affinity as was also observed in the β-bend mimicking structures (27). A further development was based on our recent discovery of the significant overlapping roles of Glu^1 and the pTyr^3 side chains in **1** (23). We found that extending the Glu^1 sidechain with an additional methylene group by using α-amino-adipate (Adi) improved the binding affinity by 6-fold in G1TE (23). In accordance with this, by substituting Adi for Glu^1 in **12**, we obtained submicromolar non-phosphorylated cyclic peptide inhibitor of Grb2-SH2 domain (**14**, IC_{50} = 0.70±0.12 μM), whereas Ala^1 mutant **12** showed only a slightly reduced binding potency (**13**, IC_{50} = 1.65±0.15 μM).

The molecular model for peptide **14** bound to Grb2-SH2 (Fig. 2) was generated based on the assumption of a β-turn for the inhibitory peptide segment, pTyr (or its mimic)-Glu-Asn-Val, as was described in the Methods Section. In the pTyr pocket the orientation and the hydrogen bonding pattern of cmPhe is consistent with previous modeling results for cmPhe containing peptides bound to Grb2-SH2 (27), and it is also in good agreement with the orientation and the hydrogen bonding pattern of cmPhe in the crystal structure of Ac-cmPhe-Glu-Glu-Ile bound to lck-SH2 domain (28). In that particular X-ray structure of lck-SH2 domain the BC loop has a different conformation, and one of the sidechain carbonyl oxygens interacts with the backbone nitrogen of Glu157, instead of the Ser156 sidechain. The latter Ser is equivalent to Ser88 in Grb2-SH2, which participates in interactions with the ligand in our case (Fig. 3a, and 3b). Adi^1 of G1TE(Adi^1, cmPhe^3) interacts with Ser90 and Lys109 of the protein, but its sidechain is located only in the periphery of the pTyr pocket, probably due to the repulsion between the side chains of Adi^1 and cmPhe^3. The lesser importance of Adi in this pTyr-mimic containing peptide is reflected by the very moderate loss of binding affinity in the G1TE (Ala^1, cmPhe^3) mutant, **13**.

G1TE(Adi[1], cmPhe[3]) blocks Grb2-SH2 domain function in cell homogenates. The Grb2 acts as an intracellular adaptor protein in transmitting activated growth factor receptor signaling by forming association with the nucleotide exchange factor, the Sos protein. In the breast cancer cell line, MDA-MB-453, the oncogenic p185^{erbB-2} receptor protein is constitutively overexpressed and phosphorylated (22). In order to evaluate the inhibitory effectiveness of our agents under near-physiological conditions, we carried out assays in cell homogenates. Peptide **14,** G1TE(Adi^1, cmPhe^3) was selected for this purpose, which showed sub-micromolar binding affinity to the Grb2 target protein in our *in vitro* SPR based competitive binding assays (Table 2). Various concentrations of the peptide were incubated with cell lysates of the breast cancer cell line MDA-MB-453, and the Grb2/p185^{erbB-2} complexes were immunoprecipitated with anti-Grb2 antibody. Western blot analysis demonstrated that peptide **14** effectively inhibited this protein association in the 0.4 to 2 μM concentration range in a dose dependent manner (Figure 3). In comparison, the Ala^3 mutant control peptide **3** was ineffective at two orders of magnitude higher concentration. These results, and our earlier report (14), demonstrate that cyclic peptides that are effective in *in vitro* SPR assays are also effective in inhibiting a key protein association process involving the oncogenic constitutively expressed erbB-2 receptor association with the Grb2 adapter protein that then mediates a key cellular activation process.

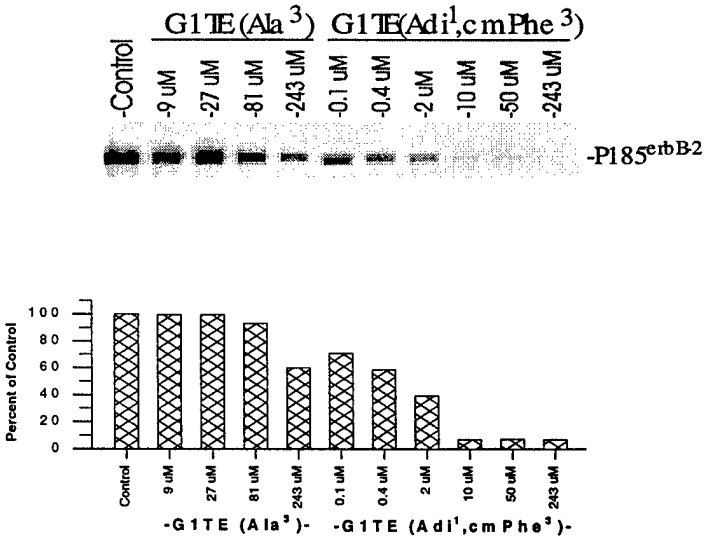


Figure 3. Inhibition of complexes formed between the Grb2 protein and GF-receptor protein p185^{erbB-2} in MDA-MB-453 cell homogenates, on treatment with peptide **3**, G1TE(Ala^3) as control at a concentration range of 9 – 243 μM, and peptide **14**, G1TE(Ala^1, cmPhe^3) at a concentration range of 0.1 – 243 μM.

Cell-based Inhibition by the Grb-2 Peptides.

Biological Cells and Cell Culture.

Cell lines were obtained from the American Type Culture Collection(Rockville,MD) and Lombardi Cancer Center, Georgetown University Medical Center. Cells were routinely maintained in improved minimal essential medium(IMEM, Biofluids, Rockville, MD) with 10% fetal bovine serum. Cultures were maintained in a humidified incubator at 37°C and 5% CO_2.

Cell Growth Assay.

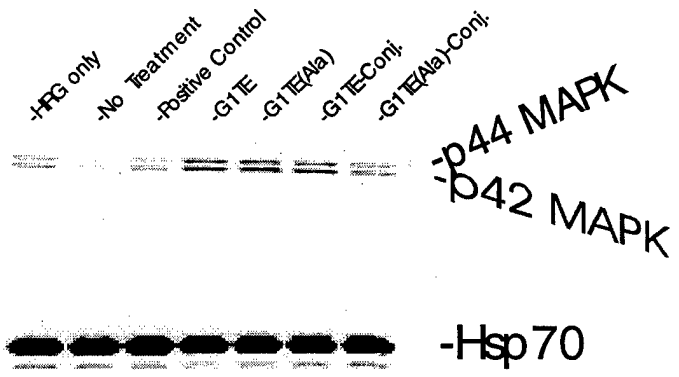
In 96 well plates, cells (MDA453 5000 cells/well, MDA-231 2000 cells/well) were grown in 10% FBS with various concentration of inhibitors. The inhibitors were added every day at the concentration indicated in the figure for five days. Then the cell growth were detected by MTT (sigma M-2128) colorimetric assay. Basically, MTT was added to the well at 0.5mg/ml final concentration and incubated with the cells at the humidified incubator (37°C and 5% CO_2) for 4 hours. After removing the medium, the stained cells inside the well were solved in 100 μl DMSO and the reduced MTT by the cell were measured at OD 570/650 nm.

Inhibition of Grb2 SH2 Domain Binding in Whole Cells.

ErbB2 overexpressing breast cancer cells, MDA-MB-453, were treated with inhibitors (30μM, 10μM, 3μM, 1μM, 0μM) for 4 h in serum-free IMEM medium. Cells were washed twice with PBS to remove inhibitor, then cell lysates were prepared using 1% Triton X-100 in PBS containing 0.2mM NaVO4. Grb2 and associated Grb2-binding proteins were immunoprecipitated from each lysate(250μg) with anti-Grb2 antibodies and collected using protein G Agarose (Roches Diagnostics GmbH #1243233). Immunoprecipitated proteins were separated by SDS-PAGE on 4-20%Tris-Glycine gradient gels (Invitrogen/Novex), and pTyr-containing proteins were detected by Western blotting using anti-phosphotyrosine antibodies (Santa Cruz #sc-7020) and visualized with ECL (Amersham). The membrane were subsequently stripped and Grb2 proteins were reprobed with an antibody recognizing the total Grb2 protein (Santa Cruz #sc-8034) as an internal immunoprecipitation control. Previous experiments have shown that a major tyrosine phosphorylated protein in these cells is the p185 erbB-2.

Inhibition of MAPK Activation in Whole Cells. The state of threoine and tyrosine phosphorylation of cellular MAP kjinase(MAPK) was determined using a polyclonal antibody specifically recognizing the phosphorylated threonine and tyrosine residues of MAPK. Briefly, MDA-MB-453 cells were plated in 6-well plates and cultured in serum-free medium overnight. Cells were treated with different concentration of various compounds for 4 h. Cell were then washed twice with ice-cold PBS and lysed in 0.5mL of lysis buffer (50mMTris-HCl,pH7.4, 150mMNaCl, 5mM $MgCl_2$, 1%Triton X-100, 5mM EDTA, 5mM EGTA, 1mM PMSF, 50μg/mL approtinin, 50μg/mL leupeptin, and 2mM sodium orthovanadate). A protein concentration was determined by BCA method (Pierce, Rockford, IL). Protein (50μg) was subjected to 4-20% SDS-PAGE gel (Invitrogen/Novex) and transferred to nitrocellulose membrane. Activation of MAP kinase was detected with a specific antibody,e.g., phospho-p44/42 MAP kinase antibody (Cell Signalling #9101S) and visualized with ECL (Amersham). The membranes were subsequently stripped and reprobed with a monoclonal antibody recognizing the Hsp-70 protein as loading control.

Effects of G1TE and Derivatives on Activation of MAP Kinase
in Human Breast Cancer Cells MDA-MB-453



Inhibition of MAP kinase activation. MDA-MB-453 cells were plated in 12-well plates and cultured in serum-free medium overnight. Cells were treated with 25uM of various peptides for 4 hours and then followed by addition of 10nM of Heregulin stimulation. Cell lysates were collected and 50 ug of protein was subject to 8-20% SDS-PAGE gel and detected with a specific phospho-p44/42 MAP kinase antibody (NEB inc.). Hsp70 served as loading control.

The following work summarized the progress made since last report and will be submitted as a manuscript to a journal.

Abstract: Distinguished from the phosphotyrosine or its mimetics containing inhibitors of Grb2-SH2, a library-based non-phosphorylated cyclic peptide ligand (termed **G1TE**) attributed its high affinity and selectivity to an additional highly favored interactions of its structural elements with the binding pocket of the protein. In this study, the functional importance of specific amino acids N-terminal to the concensus sequence **$-Y^0$-X-N^{+2}-** in **G1TE** was successfully identified. By optimizing the combination of various amino acids at positions Y-2 and Y^0, we demonstrated the significant compensatory role of position Y-2 for the absence of phosphate on Tyr^0, thus providing potent non-phosphorylated inhibitors of Grb2-SH2 domain with low micromolar or even submicromolar affinity. Biological assays on **G1TE(Gla^1)** in which the original residue of Glu^1 was substituted by γ-carboxyglutamic acid (Gla) indicated that it could inhibit the interaction between activated GF receptor and Grb2 protein in cell homogenates of MDA-MB-453 breast cancer cells at 2 μM level. More significantly, both **G1TE(Gla^1)** alone and the conjugate of **G1TE(Gla^1)** with a peptide carrier can effectively inhibit intracellular association of erbB2 and Grb2 in the same cell lines with IC_{50} of 50 μM and 2 μM respectively. The SAR studies aided by molecular modeling allows us an improved understanding of the binding mode of these novel agents, thus providing new pharmacophore models for the development of highly selective peptidomimetic variants of Grb2 antagonists.

Introduction

Growth factor receptor-bound protein 2 (Grb2) has been appreciated to be an excellent target for drugs in the treatment of cancers because of the early and central role played by Grb2 in the cellular signal transduction.[1] Grb2 is an adaptor protein comprised of a single SH2 domain flanked by two SH3 domains.[2] The SH2 domain binds to specific phosphotyrosine motifs on activated growth factor receptors such as EGFR and members of *erb*B family, or other adapter proteins such as SHC, whereas the SH3 domains associate with the nucleotide exchange factor Sos, which thus becomes activated as a positive regulator of Ras. The activated Ras triggers MAP kinase cascade which is essential for cell growth and differentiation.[3] Activated Ras oncogenes as well as overexpression of EGFR and erbB-2 have been found in a number of human cancers,[4] thus design of molecules that block the interaction between the phosphotyrosine-containing activated receptors and the Grb2-SH2 domain should interrupt the Ras signaling pathway and may promise therapeutic leads for cancers.[5]

Our approach was starting from an early discovery of a novel non-phosphorylated cyclic peptide ligand binding to Grb2-SH2. The phage-library based peptide (designated as **G1**)[6] defines a new class of SH2 domain blockers which do not require a tyrosyl phosphate or phosphonate. The lack of such a highly charged group provides a strategy to circumvent the primary drawback of current SH2 domain blockers, namely poor cell penetration and the hydrolytic lability to cellular phosphatases. G1 was identified to bind to the Grb2-SH2 protein with 10-25 μM affinity, and was comprised of a 9 a.a. long sequence motif, **E^{-2}-L-Y^0-E-N^{+2}-V^{+3}-GMY,** flanked by 2 terminal disulfide linked cysteines.[6,7] This sequence has only N^{+2} and V^{+3} in common with the consensus sequence **-pY-(L/V)-N-(V/P)** found in natural phosphopeptides bound to Grb2-SH2,[8] but **Glu**'s in position Y-2 and Y+1 are unique in this novel non-phosphorylated ligand. Our

systematic alanine scan has indicated that residues E^{-2}, Y^{0}, E^{+1}, N^{+2} and M^{+5} in G1 are required for the high affinity binding,[6,9] whereas the high affinity and specificity in phosphotyrosine containing peptide ligands of Grb2-SH2 are determined by the 3-5 residues immediately C-terminal to the phosphotyrosine.[10,8] Obviously, without pTyr or its mimics present in G1, the binding affinity is compensated for by additional well defined binding interactions within the binding pocket of the protein. These interactions are likely to involve side-chains of amino acids E^{-2}, Y^{0}, E^{+1}, N^{+2} and M^{+5}, and possibly some of the amide bond heteroatoms in G1. Also a specific conformation is required for the cyclic peptide when bound to Grb2-SH2 protein, for we have found that the ring size and the nature of cyclization linkage are important determinants for binding affinity.[11]

On the context of bioavailability, we developed an equipotent redox-stable thioether cyclized analog termed **G1TE** which effectively inhibited the association of the Grb2-SH2 protein with growth factor receptor, $p185^{erbB2}$ in cell lysates derived from the breast cancer cell line MDA-MB-453.[6] Since G1 and G1TE define a new type of SH2 domain binding motif in a pTyr independent manner, we initiated a comprehensive structure-activity studies on G1TE in order to further improve the activity and explore the salient molecular features. Contrary to the conventional efforts on the amino acids C-terminal to phosphotyrosine in the ligands of SH2 domains, our present work was focused on the residues N-terminal to the concensus sequence of $-Y^{0}$-X-N^{+2}- in the non-phosphorylated cyclic peptide. The alignment and comparison of the binding sequences between G1 and natural phosphopeptide ligands has revealed that Glu^{-2} in G1 is unique, and our earlier Ala scan studies on G1 also indicated that replacement of Glu^{-2} with Ala significantly decreased the binding affinity,[9] so, we were intrigued to explore the functional importance of **Glu^{-2}** in the interaction of G1TE and Grb2-SH2 domain. In this paper, we report the significant compensatory function of X^{-2} for phosphotyrosine, thus providing high affinity inhibitors of Grb2-SH2 domain without phosphate or phosphate mimicking functionality.

Experimental Section

Molecular Modeling. Simulations were performed with the Insight II 97/Discover 3.0 modeling package from Molecular Simulations Inc., San Diego, CA, with the cff91-force field. The X-ray structure of the KPFpYVNV peptide ligand bound the Grb2-SH2 was used as a starting geometry,[17] by replacing the turn region of this reference peptide, F-pY^{0}-V-N^{+2}-V^{+3}, with the analogous predicted turn region[9,18] of G1TE , L-Y^{0}-E^{+1}-N^{+2}-V^{+3} . The turn comprising atoms include Asn^{+2}, which possesses extraordinary specificity for Grb2-SH2 binding of phosphopeptides.[8] The turn comprising atoms, – CO(Y^{0}), E^{+1}, N^{+2}, NH(V^{+3}) –, and the protein atoms were kept fixed during the following minimization and 50 simulated annealing simulations. In each run a different random seed was used. The same minimized starting geometry was subjected to an MD simulation at 2000 K for 10 ps and consecutively cooled in 5K increments to 5 K during 195 ps. The final structure with the lowest energy was taken. The same procedure was repeated for the Adi^{-2} and Gla^{-2} mutant peptides **2** and **3**.

Binding affinity measurements using Surface Plasmon Resonance (SPR). The competitive binding affinity of ligands for the Grb2-SH2 domain protein was assessed using Biacore Surface Plasmon Resonance (SPR) methods. IC_{50} values were determined by mixing the inhibitor with recombinant GST-Grb2 SH2 protein and measuring the amount of binding at

equilibrium to an immobilized SHC(pTyr-317) phosphopeptide in a manner similar to that reported previously.[6,9]

Biological. **Cells and Cell Culture.** Cell lines were obtained from the American Type Culture Collection (Rockville, MD) and Lombardi Cancer Center, Georgetown University Medical Center. Cells were routinely maintained in improved minimal essential medium (IMEM, Biofluids, Rockville, MD) with 10% fetal bovine serum. Cultures were maintained in a humidified incubator at 37°C and 5% CO_2.

A. Inhibition Of Grb2 SH2 Domain Binding In Cell Homogenates. Cell lysates were prepared from serum-treated erbB2 overexpressing breast cancer cells (MDA-MB-453), as described previously.[6] Cell lysates were treated with G1TE(Gla^{-2}), G1TE(Gla^{-2})-carrier, or control peptide G1TE at various concentrations for 30 min, and then 500 μg of protein was immunoprecipitated with anti-Grb2 antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) and collected with protein A Sepharose. Immunoprecipitated proteins were separated by SDS-PAGE on 8-16% gradient gels (Novex, San Diego, CA, USA). pTyr-containing proteins were detected by Western blotting using anti-phosphotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY, USA) and visualized with ECL (Amersham, Arlington Heights, IL). Previous experiments have shown that a major tyrosine phosphorylated protein in these cells is the p185erbB-2, which is overexpressed as a consequence of gene amplification.[19] To evaluate the equal loading of the proteins, blots were subsequently re-probed with a monoclonal antibody recognizing the total Grb2 protein. A semi-quantitation of the Grb2 associated erbB-2 band was done with densitometer and expressed as percent of control in the untreated cells.

B. Inhibition Of Grb2 SH2 Domain Binding In Whole Cells. ErbB2 overexpressing breast cancer cells, MDA-MB-453, were treated with inhibitors (25 μM) for 3 h in serum-free IMEM medium (Gibco). Cells were washed twice with PBS to remove inhibitor, then cell lysates were prepared using 1% Triton X-100 in PBS containing 0.2 mM NaVO4. Grb2 and associated Grb2-binding proteins were immunoprecipitated from each lysate (500 μg) with anti-Grb2 antibodies and collected using protein A Sepharose. The following work-up is as the same as in cell homogenate assay.

Synthesis.

General. The PAL amide resin and Fmoc derivatives of standard amino acids were obtained from Perkin-Elmer/Applied Biosystems Division (Foster City, CA, USA). Side-chain protections of anino acids are as follows: Glu(t-Bu), Tyr(t-Bu), Asn(Trt), Cys(Trt), Lys(Boc). Fmoc-γ-carboxy-L-Glu(O*t*Bu)$_2$-OH [Fmoc-Gla(O*t*Bu)$_2$-OH], Fmoc-L-α-aminoadipic acid-δ-t-butyl ester and Fmoc-L-tyrosine(malonyl-di-OtBu)-OH were purchased from BACHEM (Torrance, CA, USA). Fmoc-L-tyrosine(PO(OH,Obzl))-OH from NOVAbiochem (La Jolla, CA, USA) was used for the synthesis of phosphotyrosine-containing analogs. Piperidine, trifluoroacetic acid (TFA), triethylsilane (TES), and chloroacetic acid were purchased from Fluka (Ronkonkoma, NY, USA). HBTU/HOBt/DIEA activation of N^{α}-protected amino acids was employed for coupling, and 20% piperidine/DMF was used for Fmoc deprotection. TFA/TES/H_2O (9.5:0.25:0.25) was used for the resin cleavage and side-chain deblocking. The crude peptides were purified to homogeneity by reverse-phase high-performance liquid chromatography (RP-HPLC). HPLC conditions: Vydac C18 column (10 x 250 mm) or Vydac C4 column (20 x 250 mm); solvent

gradient, A, 0.05% TFA in water; B, 0.05% TFA in 90% acetonitrile in water with gradient indicated below; flow rate, 2.5 mL/min for 10 mm diameter column, and 10 mL/min for 20 mm diameter column; UV detector, 225 nm. FAB-MS (unit resolution, glycerol matrix) was performed on a VG Analytical 7070E-HF mass spectrometer. The purity of products was characterized by analytical HPLC. Amino acid analysis (6N HCl, 110 °C, 24 h) was carried out at the Protein and Carbohydrate Structure Facility (University of Michigan, Ann Arbor, Michigan, USA).

General synthetic methodology of peptide ligands. Briefly, the linear peptide segments were synthesized on solid phase on a PAL amide resin, using fluorenylmethoxycarbonyl (Fmoc) chemistry based protocol (17) of an ABI 433A peptide synthesizer (FastMoc protocol) on a 0.1 mmole scale. After removing the N^{α}-Fmoc group with 20% piperidine/DMF, the resin-bound protected peptide was N-terminally chloroacetylated by $(ClCH_2CO)_2O$ which was prepared by mixing 0.5 M $ClCH_2COOH$/DCM and 0.5 M DCC/DCM for 1 h at RT. Chloroacetylation needed 6 hrs' shaking at RT, Ninhydrin Test was negative. Then the peptide was cleaved from the resin by using TFA containing 2.5% each (v/v) of triethylsilane and deionized water (2 h). For isolation of the product, two-thirds of the cleavage reagent mixture was evaporated under N_2 stream and the residue was triturated in ice-cold ether. The precipitated crude peptide was dissolved in 50 mL of water and added dropwise into 100 mL of aqueous solution, which was adjusted to pH 8~9 with triethylamine, repeatedly. Under the basic conditions the N-chloroacetylated linear peptide cyclized spontaneously by intramolecular nucleophilic displacement of the chloro group by cysteine thiol. The final product was purified by RP-HPLC, and the identity was assessed by amino acid and mass spectral analyses.

Cyclo-(CH_2CO-Glu-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys)-amide (1). HPLC gradient 20-80% B over 30 min, R_t = 12.8 min) to provide G1TE in overall yield of 30%. FAB-MS $(M+H)^+$ 1258.7 (calcd 1259.5). Amino acid analysis: Asp + S-CM-Cys (1 each), Val (1), Leu (1), Glu (2), Gly (1), Tyr (2), Met (1).

Cyclo-(CH_2CO-Ala-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys)-amide (2). Yield

Cyclo(CH_2CO-Gla-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys)-amide (6). The product was purified by RP-HPLC (gradient 20-70% B over 27 min, R_t = 11.4 min) to provide G1TE (Gla^1) in overall yield of 30%. FAB-MS $(M+H)^+$ 1303.4 (calcd 1303.5). Amino acid analysis: Asp + S-CM-Cys 1.51(1 each), Val 1.00(1), Leu 1.23(1), Glu + Gla 1.88(1 each), Gly 1.18(1), Tyr 1.83(2), Met 0.85(1).

Cyclo(CH_2CO-Adi-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys)-amide (5). It was prepared analogously to G1TE(Gla^1). RP-HPLC R_t = 13.6 min (gradient 20-70% B over 27 min) in overall yield of 40%. FAB-MS $(M+H)^+$ 1273.4 (calcd 1273.5). Amino acid analysis: Asp 0.43(1), S-CM-Cys 0.98(1), Adi 0.97(1), Val 1.00(1), Leu 1.21(1), Glu 1.09(1), Gly 1.13(1), Tyr 1.87(2), Met 0.93(1).

Chemistry

The thioether cyclized peptides were synthesized on the solid-phase peptide synthesis in Fmoc chemistry as outlined in Scheme 1.[12] Various structures of amino acids were built into positions Y-2 and Y-0 in G1TE as depicted in Figure 1 and Figure 2. The building block L-

carboxymethyl-phenylalanine (cmPhe) was prepared with orthogonal O-*t*Bu protection suitable for Fmoc-based chemistry as described previously.[13]

Results and Discussion:

Compensatory and overlapping function between positions Y^{-2} and Y^{0} in G1TE bound to Grb2-SH2 confers high affinity binding. Lead peptide G1TE (**1**) and its various analogs substituted in position Y-2 were synthesized and the Grb2-SH2 domain inhibitory activity was assessed by using Biacore SPR methodology [Table 1]. Our earlier Ala scan studies with the non-phosphorylated disulfide linked cyclic peptide **G1** showed that replacement of Glu^{-2} with Ala ruined the binding affinity to Grb2-SH2.[9] The Ala^{-2} substituted analog of **G1TE** in the current study confirms the finding (**2**, IC_{50} = 100 μM). Predictably, phosphorylation of Tyr^{0} in G1TE greatly improved the potency by a factor of 150 fold (**3**, IC_{50} = 0.13 μM), which was in accord with our initial finding that G1 peptide requires a YXN sequence similar to that found in natural pTyr-containing ligands, and phosphorylation of the tyrosine increases G1 inhibitory activity.[6] Surprisingly, when Glu^{-2} was replaced by Ala in the pTyr containing peptide **3**, an even higher affinity was observed (**4**, IC_{50} = 0.023 μM), suggesting that the polar side-chain of Glu^{-2} competes for the conformational space occupied by the $pTyr^{0}$ phosphate functionality. From this, further extending the side-chain of Glu^{-2} in G1TE with a CH_2 moiety (X^{-2} = α-aminoadipic acid, **5**) or attaching one more carboxyl group to the Glu^{-2} side-chain (X^{-2} = γ-carboxyglutamic acid, **6**) results in a significant increase in the inhibitory potency by 6-fold (**5**, IC_{50} = 3.45 μM) and 31-fold (**6**, IC_{50} = 0.64 μM) respectively. These results confirm the similar and overlapping roles of Glu (Adi, Gla) in position Y-2 and of the $pTyr^{0}$ side-chains.

Figure 1. The structures of various amino acids built into position Y-2.

Table 1. Grb2-SH2 Domain Inhibitory Activity of the Peptides **1-10**[a] with variations in X^{-2}.

O=C-NH-**X^{-2}**-L-(p)**Y^{0}**-E-N-V-G-M-Y-C-Amide S	Cpd	X^{-2}	Y^{0}	IC_{50} (μM)
	1	Glu	Tyr	20 ± 5
	2	Ala	Tyr	100 ± 20
	3	Glu	pTyr	0.13 ± 0.01
	4	Ala	pTyr	0.023 ± 0.01
	5	N-α-aminoadipic acid (Adi)	Tyr	3.45± 0.15
	6	γ-carboxyglutamic acid (Gla)	Tyr	0.64 ± 0.10
	7	pTyr	Tyr	27.5 ± 6.5
	8	pTyr	Ala	At 1000 μM, 37% inhibition

Figure 2. The structures of the substituents at position Y^{0}.

	9	malonylTyr	Tyr	64.5 ± 2.5
	10	Cit	Tyr	330

[a]Competitive binding assays with SHC phosphopeptide, DDPSpYVNVQ, using surface plasmon resonance on a BIAcore 2000 instrument as reported earlier[3]. The results represent mean value of at least two independent experiments and are expressed as the concentration at which half-maximal competition was observed (IC_{50}).

The side chains in position Y^{-2} can not fully occupy the $pTyr^0$ binding pocket in the Grb2-SH2 protein. Further modifications were designed to test if the side chain in position Y-2 can fully occupy the pTyr binding pocket in the Grb2 SH2 protein or if the two sites (positions Y-2 and Y^0) independently provided binding affinity. We incorporated highly negative charged residue pTyr, and the pTyr mimetic O-malonyl-tyrosine (malTry) into the Y-2 position (**7** and **9**). Contrary to the enhancing binding effect when built into position Y^0, these substitutions in position Y-2 turn out potency loss, i.e., IC_{50} = 27.5 μM for **7** and IC_{50} = 64.5 μM for **9**. Moreover, we synthesized an anolog with both substitutions of $pTyr^{-2}$ and Ala^0 (peptide **8**), as a comparison with the most potent peptide **4** which has Ala^{-2} and $pTyr^0$ (**4**, IC_{50} = 0.023 μM), but the inhibitory activity was collapsed (for **8**, at 1000 μM, 37% inhibition observed). These results strongly suggest that the side-chains in position Y-2 does provide a compensatory role for the lack of phosphate on Tyr^0, but does not fully occupy the pTyr binding pocket in the protein. On the other hand, the rigid aromatic moiety, shared by pTyr and malonylTyr, may be responsible for the loss of compensatory interaction with $pTyr^0$ due to a disfavored positioning of the malonyl or phosphate terminal groups. More likely, amino acids in position Y–2 that possess flexible alkyl chains can orient the polar carboxyl groups to an optimal position for binding interaction with the protein.

Optimizing the combination of amino acids in position Y-2 and Y^0 produces potent inhibitors of Grb2-SH2. Since positions Y^{-2} and Y^0 share an overlapping function in the Grb2-SH2 binding, we suppose that the optimal combination of the substitutions in the two positions would produce high affinity antagonist of Grb2-SH2. As shown in Figure 2, we incorporated five different residues into position Y^0, i.e. the original Tyr, phosphotyrosine (pTyr), homotyrosine (hTyr), N-α-aminoadipic acid (Adi) and carboxymethyl-phenylalanine (cmPhe). On the basis of our preceding discovery about the distinct effect of various Y^{-2} substitutions on the binding potency, we designed and synthesized a series of G1TE analogs with various substitutions on position Y^0 first then plus selective substitution on position Y-2 to achieve an optimal combination. Three amino acids were chosen to incorporate into position Y-2 besides the original one i.e. Glu, Ala, Adi. As shown in Table 2, when the side chain of Tyr^0 was extended with one CH_2 moiety (hTyr), the binding affinity was destroyed (**11**, at 1000 μM, 44% inhibition observed). But additional incorporation of Adi in position Y-2 greatly saved the potency (**12**, IC_{50} = 190 μM). However, in the substitution of Adi^0, the lacking of the phenyl group in position Y^0 results in activity eliminated (**13**, at 1500 μM, 25% inhibition), even the favorable substitution of Adi in position Y^{-2} combined does not help much (**14**, IC_{50} = 1400 μM). Not surprisingly, the combination of Ala^{-2} with Adi^0 substitutions absolutely abrogated the binding affinity (**15**, inactive). Similarly, but to the other extreme, the high affinity binding of $cmPhe^0$ containing peptide is not much sensitive to the variations in position Y-2 though slight increase or decrease occurs when Glu^{-2} (**17**, IC_{50} = 1.15 μM) is replaced by Adi (**18**, IC_{50} = 0.7 μM) or Ala (**19**, IC_{50} = 1.65 μM). By comparing these data with that of variations in position Y-2 alone (peptides **1**-**4**),

we can suggest that the compensatory function of position Y^{-2} varies with the different structures of the amino acids built into position Y^0. Generally, there are 3 types of Y^0 substitution effects on the compensatory function of position Y-2. When Tyr or hTyr is in position Y^0 which provides phenyl group but lacks the negative charge, the acidic side chain in the compensatory position of Y-2 can provide a significant enhancement in Grb2-SH2 domain binding potency. When pTyr is present in position Y^0 which carries bulky and highly charged phosphate, the acidic side chain in position Y-2 is unfavored and Ala substitution showed improved activity. When the pTyr mimic cmPhe is built into the position Y^0 which carries less bulky but still double charged carboxyl groups, the side chain in position Y-2 exhibited a slight influence on its binding. However, when Tyr is substituted with Adi which bears alkyl carboxyl side chain and lacks the phenyl group, the acidic side chain in the position Y-2 can not compensate the absence of phosphate and phenyl functionalities in position Y^0. These results further confirm the finding of the compensatory and overlapping functions position Y-2 provides for Tyr^0 in this novel non-phosphorylated ligand binding to Grb2-SH2.

Table 2. Grb2-SH2 Domain Inhibitory Activity of the Peptides **11-19** with variations at position Y^0 and the compensatory postion X^{-2}.

Compound	X^{-2}	X^0	IC_{50} (μM)
1	Glu	hTyr	At 1000 μM, 44% inhibition
2	Adi	hTyr	190 ± 40
3	Glu	Adi	At 1500 μM, 25% inhibition
4	Adi	Adi	1400 ± 100
5	Ala	Adi	inactive
6	Lys	Adi	inactive
7	Glu	cmPhe	1.15 ± 0.05
8	Adi	cmPhe	0.70 ± 0.12
9	Ala	cmPhe	1.65 ± 0.15

G1TE(Gla^{-2}) and G1TE(Gla^{-2})-carrier conjugate effectively inhibit the association of oncogenic ErbB-2 and Grb2 in whole cell assay as well as in cell homogenates assay. Biacore data present in Table 1 and 2 reflect binding of isolated Grb2 SH2 domain fusion protein to a reference phosphopeptide SHC(pY317). This type of data is a useful indicator of protein-inhibitor interaction. However, in physiological contexts, binding interactions occur between full Grb2 protein (which consists of an SH2 domain and two SH3 domains[2]) and phosphorylated proteins, which include SHC and erbB-2 growth factor receptor cytoplasmic domains. Therefore, in order to evaluate the effectiveness of synthetic peptides to inhibit the interaction of native Grb2 protein with activated cellular growth factor receptor, cell homogenates assay was conducted. MDA-MB-453 cells were used, which are derived from a human breast cancer where there is amplification of *erb*B-2 gene.[5] Peptide **6** was selected for this purpose, which showed submicromolar binding affinity to the Grb2 target protein in our in vitro SPR-based competitive binding assays (Table 1). We incubated the active peptide **6** with cell lysates of the MDA-MB-453 cell lines which overexpress the oncogenic p185(erbB-2) receptor protein. As shown in **Figure** 3, Peptide **6** was effective in inhibiting protein/protein association in a dose dependent manner, at the half maximal concentration of 2.0 ± 0.8 μM, whereas the prototype G1TE served

as a reference. Furthermore, to facilitate the cellular internalization of the synthetic inhibitor, a peptide carrier with the sequence of **AAVALLPAVLLALLAP** was conjugated to the C-terminal of peptide **6**. The location of the carrier sequence in the N-termimi or C-termini has been revealed to make no difference for the peptide import function.[14] Encouragingly, even in the presence of the hydrophobic16-mer carrier, the inhibitory activity of peptide **20** keeps intact in the cell homogenate assay (**20**, IC_{50} = 2.0 μM).

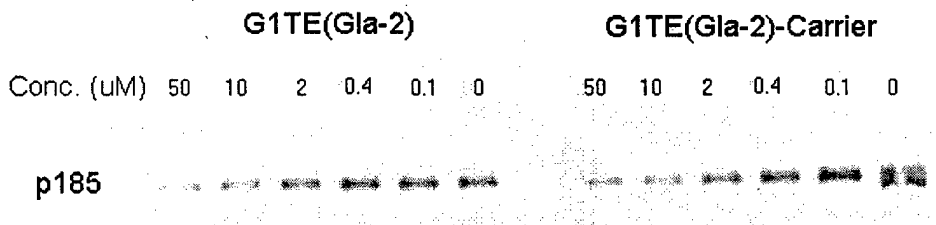


Figure 3. Inhibition of complexes formed between the Grb2 protein and GF-receptor protein P185$^{erb\text{B-}2}$ in MDA-MB-453 cell homogenates, on treatment with peptide **6**, G1TE(Gla-2) at a concentration range of 0.1 – 50 μM, and the conjugate peptide **20** at the same concentration range (see Experiment for details).

On the basis of cell lysate assay, we further examined the inhibitory potency of active peptide **6** and its conjugate **20** in whole cells in which inhibition of binding of full length Grb2 to native erbB-2 was measured following treatment of cells with synthetic inhibitors. In cellular systems ligands must cross cell membranes prior to interacting with targets. Shown in **Figure** 4 are results from these assays. As expected, the hydrophobic carrier enhances cellular membrane penetration, thus the conjugate peptide **20** exhibited significant intracellular inhibition of Grb2-SH2 binding to erbB-2 receptor. Surprisingly, the inhibition of cognate Grb2 SH2 domain binding was also observed on peptide **6** with IC_{50} of 50 μM.

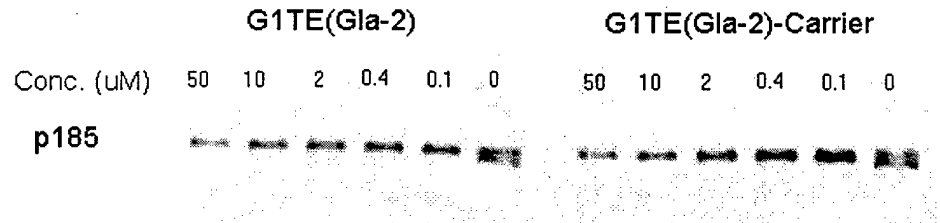


Figure 4. Effects of inhibitors on interaction of Grb2 with tyrosine phosphorylated P185$^{erb\text{B-}2}$ in human breast cancer cells MDA-MB-453 as described in Experimental Section. MDA-MB-453 cells were plated in 100 mm dishes cultured in DME with 10% FBS medium overnight. Cells were treated with inhibitors (25 μM) for 3 h, washed, solubilized and 500 μg of protein immunoprecipitate was treated with anti-pTyr antibody. Co-immunoprecipitated pTyr-containing P185$^{erb\text{B-}2}$ was detected using pTyr antibody (Py-99) and immunoblotting. Western blotting with Grb2 Mab was done as a control.

Molecular modeling confirms the overlapping function of position Y-2. Thermodynamic,[15] NMR[16] and X-ray[17] analyses have shown that pTyr containing peptides bind to SH2 domains in a β-turn conformation in the region of X^{-1}pTyr-X^{+1}-Asn- in these ligands. Also, it has been found consistently that the negatively charged Tyr-phosphate oxygens form key binding interactions with the Arg67 (αA-helix) and Arg86 (βC-strand) residues of the protein and the three Ser residues 88, 90 and 96 of the protein, and the consensus amino acid Asn^{+2} of the ligands forms well conserved interactions as well. We have docked G1TE (**1**) and G1TE(Gla^{-2})(**3**) into the Grb2-SH2 3D binding pocket, assuming that Tyr^{0} and Asn^{+2} occupy near proximal binding sites before dynamic simulation, as found previously

for phosphopeptides.[17] The energy minimized adduct structures display a possible mode of binding for G1TE [Figure 5], in which the Glu^{-2} carboxyl group together with Tyr^{0} interacts with both Arginines and Serines 88 and 90 of the binding pocket. The docking results for G1TE(Gla^{-2}) confirm that γ-carboxyglutamic acid in position Y–2 is favored over Glu in that position, because the second carboxyl group can undergo additional interactions in the pTyr binding pocket.

Conclusions: In this study, we have successfully explored the functional importance of N-terminal Glu in the position Y-2 of the non-phosphorylated ligand, cyclo(CH_2CO-Glu^{-2}-Leu-Tyr^{0}-Glu-Asn-Val-Gly-Met-Tyr-Cys)-amide with respect to its binding interactions with the Grb2-SH2 protein, and determined that the carboxyl side chain in that position provides compensation for the absence of phosphate on Tyr^{0}. When extending the side-chain of Glu^{-2} by one CH_2 moiety (X^{-2} = α-aminoadipic acid, **2**), or adding one more carboxyl group to the side-chain (X^{-2} = γ-carboxyglutamic acid, **3**), the resulting modified carboxyl side-chain(s) provide for more favorable ionic interactions with the guanidino functionalities of Arg67 and Arg86 in the protein Grb2-SH2. This results in binding improvement by 8-fold (low micromolar affinity for **2**) and 30-fold (submicromolar affinity for **3**), respectively. These new agents are among the most potent non-phosphorous- and non-tyrosinephosphate-mimic containing SH2 domain inhibitors yet reported. The reverse effects of Ala substitution for Glu^{-2} in tyrosine-containing G1TE and phosphotyrosine-containing G1TE(pY) further prove the similar and overlapping roles of Glu in position Y–2 and the $pTyr^{0}$ side chains. On the other hand, the potency reduction of the malonyltyrosine and phosphotyrosine in position Y-2 can be attributed to the non-optimal positioning of the carboxyl and phosphate groups because of their rigid substructures. These results indicate the significance and the specific compensatory role of position Y-2 in the non-phosphorylated peptide inhibitor, cyclo(CH_2CO-X^{-2}-Leu-Tyr^{0}-Glu-Asn-Val-Gly-Met-Tyr-Cys)-amide, and thus advance the design of potent non-phosphorylated antagonist of Grb2-SH2 domain for new anticancer therapeutics.

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KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of **key** research accomplishments emanating from this research.

- Design and synthesis of cyclic and other modified Geb2 peptide inhibitors with improved activities over G1TE;
- Biochemical and biological studies of the modified Grb2 peptide inhibitors;
- Provided the proof-of-concept for the small molecule inhibitor studies of Grb2 interruption and also the success of funding from Komen Foundation for Breast Cancer.
- Provided more potent peptides with further cell based inhibition activities.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes to include:

– manuscripts, abstracts, presentations;

1. Long, Y.-Q.; Lung, F.-D.T.; Voigt, J.H.; Yao, Z.-J.; Burke, Jr., T.R.; **Yang, D.**; Luo, J.H.; Guo, R.; King, C.R.; Roller, P.P. High affinity nonphosphorylated cyclic peptide inhibitors of Grb2-SH2 / growth factor receptor interactions. In Peptides for the New Millennium (Proceedings of the 16th American Peptide Symposium). Fields, G.B.; Tam, J.P. and Barany, G. (Eds.); Kluwer Academic Publishers, Dordrecht, The Netherlands, **2000**, 568-570.
2. Long, Y.-Q.; Voigt, J.H.; Luo, J.H.; Guo, R.; Roller, P.P; **Yang, D.** Overlapping function of amino acids in position-2 and Y-0 conferring high-affinity to nonphosphorylated peptide ligands of GRB2-SH2 domain. Intended for J. Med. Chem. In Preparation.
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*Those are related publications in the same target use small molecule approaches.

– patents and licenses applied for and/or issued;

An international patent application was filed in 2001 and so was a domestic one. A final paperwork is being done to submit the domestic application:
U.S. Patent Application No. 09/998,350. "Redox-Stable, non-phosphorylated cyclic peptide inhibitors of SH2 domain binding to target protein, conjugates thereof, compositions and methods of synthesis and use.' DHHS reference: E-081-99/1
Inventors: Peter P. Roller, Ya-Qiu Long, Feng-Di T. Lung, C. Richter King, and **Dajun Yang**.

- degrees obtained that are supported by this award;

  Not applicable.

- development of cell lines, tissue or serum repositories;

  Not applicable.

- informatics such as databases and animal models, etc;

  Not applicable.

- funding applied for based on work supported by this award;

  Susan G. Komen Breast Cancer Foundation

  Principal Investigator

  Title: Grb2 Targeted Therapeutics for Breast Cancer

  1999-2001, annual direct $100,000.00, annual indirect $25,000

- employment or research opportunities applied for and/or received on experiences/training supported by this award.

  Not Applicable.

CONCLUSIONS: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the annual and final reports.

By incorporating a series of various structurally relevant amino acids in position Y^3 of the cyclic peptide G1TE, we explored the functional importance and structural requirement for Tyr^3. Phenyl moiety and polar group with specific orientation in position Y^3 are particularly required for high affinity binding of G1TE to its cognate protein, the Grb2-SH2 domain. Substitutions of pTyr mimetics for Tyr^3 provide low micromolar to submicromolar affinity inhibitors. The combination of cmPhe replacement in position Y^3 and Adi substitution in position E^1 results in a potent non-phosphorylated cyclic peptide antagonist of Grb2-SH2 domain with $IC_{50} = 0.70 \pm 0.12$ μM. When pTyr containing peptides bind to various SH2 domains of proteins, the pTyr binding pocket provides the driving force for binding affinity. The Grb2-SH2 domain is unique, in that ligands binding to it are required to possess turn conformations, such that pTyr, and Asn or its mimic in the pTyr + 2 position, are required to fit into specific receptor sites. The merit of the G1TE family of nonphosphorylated cyclic peptides is that for high affinity binding they require additional well defined conformational space, in order to accommodate the requisite interactions with the acidic sidechains of Glu or Gla in the Tyr – 2 position of the peptide, as our previous work demonstrated. These results provide a better understanding of the molecular binding mode of this novel non-phosphorylated cyclic peptide ligand, and suggest new strategies for designing potent and specific non-phosphorylated inhibitors of Grb2-SH2 domain. Very recently the

discovery of a number of non-phosphorylated cyclic peptides was disclosed, using phage library methodology, that showed low micromolar binding affinities to Grb2-SH2. These structural variants have in common with our peptides the strong selection of Glu at the Tyr – 2 position. These additionl variants will provide additional possibilities for rational design of adventageous peptides and peptidomometics.

Furthermore, in recent study, we have successfully explored the functional importance of N-terminal Glu in the position Y-2 of the non-phosphorylated ligand, cyclo(CH_2CO-Glu^{-2}-Leu-Tyr^{0}-Glu-Asn-Val-Gly-Met-Tyr-Cys)-amide with respect to its binding interactions with the Grb2-SH2 protein, and determined that the carboxyl side chain in that position provides compensation for the absence of phosphate on Tyr^0. When extending the side-chain of Glu^{-2} by one CH_2 moiety (X^{-2} = α-aminoadipic acid, **2**), or adding one more carboxyl group to the side-chain (X^{-2} = γ-carboxyglutamic acid, **3**), the resulting modified carboxyl side-chain(s) provide for more favorable ionic interactions with the guanidino functionalities of Arg67 and Arg86 in the protein Grb2-SH2. This results in binding improvement by 8-fold (low micromolar affinity for **2**) and 30-fold (submicromolar affinity for **3**), respectively. These new agents are among the most potent non-phosphorous- and non-tyrosinephosphate-mimic containing SH2 domain inhibitors yet reported. The reverse effects of Ala substitution for Glu^{-2} in tyrosine-containing G1TE and phosphotyrosine-containing G1TE(pY) further prove the similar and overlapping roles of Glu in position Y–2 and the $pTyr^0$ side chains. On the other hand, the potency reduction of the malonyltyrosine and phosphotyrosine in position Y-2 can be attributed to the non-optimal positioning of the carboxyl and phosphate groups because of their rigid substructures. These results indicate the significance and the specific compensatory role of position Y-2 in the non-phosphorylated peptide inhibitor, cyclo(CH_2CO-X^{-2}-Leu-Tyr^{0}-Glu-Asn-Val-Gly-Met-Tyr-Cys)-amide, and thus advance the design of potent non-phosphorylated antagonist of Grb2-SH2 domain for new anticancer therapeutics.

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APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples of appendices include journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

We will mail these appendices separately.

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